Biomarkers of Oxidative Stress and Genotoxicity in Livers of Field-Collected Brown Bullhead

LONG-TERM EFFECTS
OF DREDGING OPERATIONS PROGRAM

PURPOSE: This technical note describes the application of a suite of biomarker assays to samples of brown bullhead liver tissues taken from two locations in Ohio. One was a river reach that had historically been severely impacted by the effluents of a coking plant; the other is regarded as relatively pristine, and is a freshwater estuarine research preserve. The objective of the study was to investigate whether differences in biomarker responses in the fish livers could be detected and related to the pollution histories of the two locations, and to the condition of the fish found in them.

BACKGROUND: Many of the major contaminants of sediments found in industrialized waterways are tumorigenic in laboratory studies and some of these are classified as carcinogens in animals or humans. For that reason, research was undertaken at the U.S. Army Engineer Waterways Experiment Station (WES) in 1990 to identify and develop methods that can be used to assess the genotoxic and carcinogenic potential of dredged sediments. The results of this work have been reported in previous technical notes of this series (EEDP-01-23, and -40, EEDP-04-20, -24, -25, and -26).

In previous studies, microbial methods were evaluated for their ability to characterize the mutagenicity of sediment extracts. The utility of cultured mammalian cell lines for identification of effects on specific enzyme systems and the cytotoxicity of extracts was examined. Following this work, assays were developed and applied to earthworms exposed to contaminated substrates in the laboratory. In the work reported here, the assays previously developed were applied to fish collected at a location contaminated primarily with polyaromatic hydrocarbons (PAHs) and to fish collected from a nearby location considered to be free from industrial influences.

INTRODUCTION

Metabolism and Oxidative Stress. Neutral foreign compounds are detoxicated in eukaryotes primarily by metabolism and excretion of the conjugated metabolite. In most cases, the xenobiotic is bioactivated by insertion of molecular oxygen catalyzed by mixed-function oxidases of the cytochrome P-450 system (Phase I), enabling it to bind with an endogenous substrate such as glutathione or sulfate (Phase II) (Figure 1, [1]). Some chemical classes such as nitro- or azo-aromatics are reductively bioactivated by the P-450 system, and others, e.g., esters, are hydrolyzed. In all cases the increased water solubility of the product facilitates excretion. However, bioactivation can also result in formation of reactive intermediates capable of covalently binding with macromolecules such as enzymes or nucleic acids. A classic example of this is bioactivation of the PAH compound benzo[a]pyrene (B[a]P) in a series of oxidative steps to form the highly carcinogenic electrophile B[a]P-7,8-dihydrodiol-9,10-epoxide.

Bioactivation may also result in a compound capable of undergoing redox cycling, or in the case of compounds such as quinones, aromatic nitro compounds, or hydroxylamines, the parent compound

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[1] Phase I metabolism (including oxidation by cytochrome P-450) may form reactive metabolites, which can either react with cellular components or be detoxified by Phase II metabolism (such as a conjugation to glutathione by glutathione-s-transferases (GST)). [2] Phase I metabolism can also result in the formation of redox cycling compounds. [3] Redox cycling compounds can be reduced by flavoproteins to form radical metabolites. [4] To complete the cycle, the radical metabolite autooxidizes forming the superoxide anion (O_2•-) and regenerating the parent compound. [5] In the presence of iron, the superoxide anion undergoes the Haber-Weiss reaction, forming the highly reactive hydroxyl radical (HO•). [6] Superoxide can be degraded by superoxide dismutase (SOD) forming hydrogen peroxide (H_2O_2). [7] In the presence of iron, H_2O_2 can undergo Fenton's reaction forming molecular oxygen and hydroxyl radical. [8] H_2O_2 is degraded by catalase (CAT), or by glutathione peroxidase (GSX) which utilizes reduced glutathione (GSH) as a proton donor. [9] Glutathione reductase (GR) regenerates the protective free thiol (GSH) from glutathione disulfide (GSSG). Details in text.
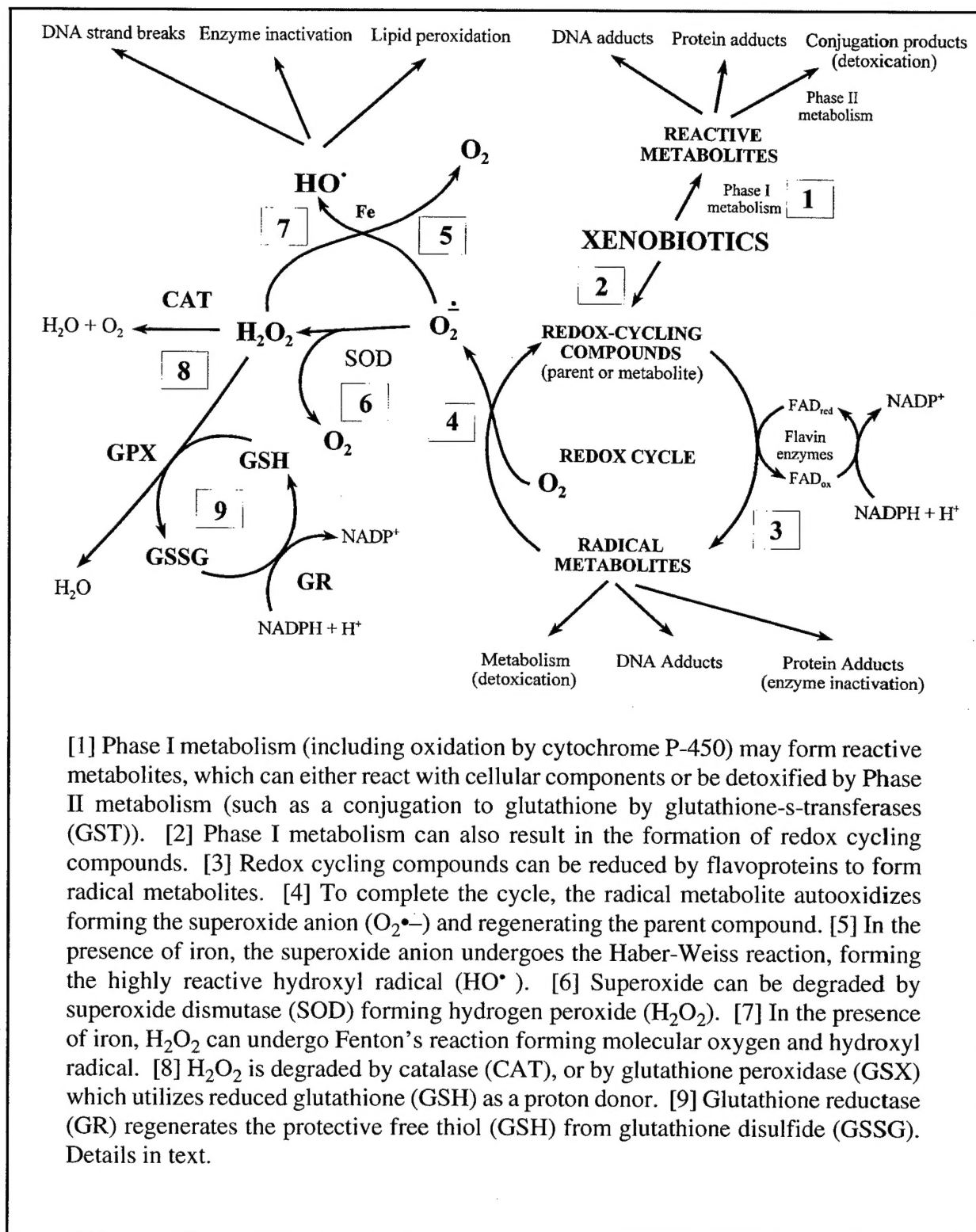
Figure 1.   Overview of routes by which xenobiotics may result in toxicity and how they may be detoxified. (Adapted from Kappus (1987))

itself may redox cycle (Figure 1, [2]). Redox cycling is a secondary metabolic pathway in which the parent compound is reduced by flavoproteins, transforming it into a radical metabolite (Figure 1, [3]). The radical metabolite is capable of reacting directly with molecular oxygen, producing the superoxide anion $O_2^{\cdot-}$ and regenerating the parent compound (Figure 1, [4]). The cycle may be repeated with each turn producing another superoxide anion until the metabolite radical is either adducted to an endogenous macromolecule (e.g., DNA or an enzyme) or is conjugated and eliminated (detoxified). Some compounds can form reactive metabolites by both the cytochrome P-450 and redox cycling routes. For example, in brown bullhead B[a]P-quinones represent a major portion of the Phase I oxidation products (Sikka, Rutkowdki, and Kandaswami 1990; Yuan, Kumar, and Sikka 1997) that are capable of either redox cycling or Phase II conjugation.

The superoxide radical itself is a poor oxidant and will not result in oxidative damage to the organism, but in the presence of iron it can undergo the Haber-Weiss reaction, forming molecular oxygen and the extremely reactive hydroxyl radical $HO^{\cdot}$ (Figure 1, [5]). The enzyme superoxide dismutase (SOD) can reduce the formation of $HO^{\cdot}$ from superoxide radicals by catalyzing the formation of hydrogen peroxide (H_2O_2) instead (Figure 1, [6]). Hydrogen peroxide is also capable of forming $HO^{\cdot}$ in the presence of iron through Fenton's reaction (Figure 1, [7]). However, the enzymes catalase (CAT) and glutathione peroxidase (GPX) normally function as a defense against this reaction by reducing H_2O_2 to water (Figure 1, [8]). In the case of GPX, reduced glutathione (GSH) acts as a proton donor, resulting in the formation of glutathione disulfide (GSSG) (Figure 1, [9]); the enzyme glutathione reductase (GR) restores GSSG to the protective free thiol.

The importance of redox-cycling as a bioactivating process is that each mole of xenobiotic can potentially produce multiple moles of active oxygen species and result in oxidative stress to the organism. Damage begins to accumulate when the formation of active oxygen species exceeds the organism's capacity to neutralize them. This includes mutations that may eventually lead to the formation of cancers. Organisms normally cope with increased oxidative stress by increasing the levels of protective enzymes and antioxidants. For this reason enzymes such as CAT, SOD, GPX, GR, and GST and the substrate GSH may serve as biomarkers. Comparing the amounts of these biomarkers in fish collected from impacted and clean areas may provide insights into the quality of the water and sediments and the probability of adverse responses in fish exposed to them, including the probability of developing cancers.

Biomarkers of Oxidative Stress in Fish Livers. The brown bullhead, *Ameriurus nebulosus*, is susceptible to pollutant-induced neoplasia, and has been used as a sentinel organism for pollutants in waterways (Baumann and Harshbarger 1985, 1995). Brown bullheads develop tumors in response to pollutant exposure more readily than the channel catfish, *Ictalurus punctatus*, and this difference has been related to the ability of channel catfish liver microsomes to produce considerably lower levels of benzene ring diols than do the liver microsomes of brown bullhead — a Phase I bioactivation process (Yuan, Kumar, and Sikka 1997). Others have related the differences in susceptibility to cancer of the two species to the higher capacity of the brown bullhead to produce redox cycling metabolites, which in turn produce oxidizing radicals and also cause reductions in the levels of antioxidants and protective enzymes (Di Giulio et al. 1995). It seems likely that since the differences in both Phase I metabolism and in redox cycling favor tumor production in the brown bullhead, both processes contribute to the high incidence of cancer in this species.

The incidence and nature of tumors in brown bullhead livers collected in the wild have been well characterized. High incidences of liver neoplasms have been related to exposure to chemical contaminants, primarily PAHs (Baumann and Harshbarger 1985, 1995). In the study reported in this technical note, brown bullheads were collected at previously monitored locations. Multiple biomarkers were measured in portions of the livers of the fish in an attempt to correlate responses at the biochemical level with differences in condition of the fish at the two collection sites.

METHODS AND MATERIALS

Sample Collection and Storage. Adult male and female brown bullhead were collected in May 1996 at two locations in Ohio: (1) on the Black River (BR), Lorain County, near a coking plant outfall pipe; and (2) at Old Woman Creek (OWC) National Estuary, Huron County. Twenty-four fish were taken at BR (14 male, 10 female) and 30 fish were taken at OWC (22 male, 8 female). The fish were collected from fyke nets deployed on the bottom of the respective water bodies for approximately 24 hr. The nets were then lifted and captured fish were transferred to a live box for transport to the laboratory. In the laboratory the fish were anesthetized, weighed, measured, sexed, hematocrits were taken, inspected for external tumors and other lesions, and dissected to remove the liver and other organs. Liver sample splits were placed in 1.5-ml cryovials and frozen in liquid nitrogen for transport. Subsamples were conveyed to the Department of Pathology, George Washington University, Washington, DC, for histopathological analysis, and to WES for biomarker assays. At WES, the samples were partially thawed and cut into ~ 0.4-g aliquots for biomarker analysis; aliquots were then refrozen in a -80 °C freezer until analyzed.

Biomarker Assays. Biomarkers included a suite of oxidative stress enzyme assays (SOD, CAT, GPX, and GR), the Phase II enzymes referred to collectively as glutathione S-transferases (GST), total and oxidized glutathione (TGSH and GSSG), ethoxyresorufin-*O*-deethylase (EROD) and the alkaline unwinding assay for DNA single strand breaks (ssDNA). All assays were conducted on 96-well plates to increase speed and efficiency of performance with the exception of the ssDNA assay, which was conducted by the method of Shugart (1988). EROD was measured as described in Hahn et al. (1993). Glutathione was measured using the methods of Baker, Cerniglia, and Zaman (1990), while SOD and CAT assays were conducted with the methods of Mishra and Mishra (1996) and Cohen, Mimi, and Vivian (1996), respectively. Assays for GPX (both Se-independent and Se-dependent), GR, and GST were adapted to the 96-well plate format from classical spectrophotometric methods (Flohe and Gunzler 1984, Cohen and Duvel 1988, Habig and Jakoby 1981). Details of performance of the assays are available by contacting one of the authors.

Statistical Analysis. Data were subjected to analysis of variance after first testing assumptions of normality using Shapiro-Wilk's test and equality of variances using Levene's test. In most cases assumptions were met using untransformed data, but in some instances conversion to rankits was necessary. Two-factor anova was used, with location as the primary factor of interest and sex as a secondary factor. When location-by-sex interaction was significant, differences in location were tested separately for each sex. Analyses of covariance were performed using the same primary and secondary factors, and using bullhead age or relative liver weight (RLW) as the covariate. Spearman's Rank Order Correlation was also used to compare RLW and the presence or absence of surficial tumors. T-tests or Mann-Whitney Rank Sum tests were used for some two-parameter comparisons.

RESULTS AND DISCUSSION: In the interval since closure of the coking plant responsible for contamination of the Black River sediments at the collection site, surveys have shown the PAH contamination of the fish and sediments to have greatly abated. Concentration of total PAHs in sediments near the plant outfall was reported to be 1,096 µg/g dry weight in 1980 and by 1987 had declined to only 4.27 µg/g. Liver pathologies in the fish paralleled the PAH concentrations, with 39 percent of 3- to 4-year-old fish having cancerous neoplasms in 1982, and 10 percent having cancers in 1987 (Baumann and Harshbarger 1995).

In the present investigation, two fish at each site had liver neoplasms. One of the OWC fish was described as having a single tiny focus of hepatocellular alteration, and another had a single cholangiocarcinoma. One of the BR fish had multiple cholangiocarcinomas and one hepatocellular carcinoma, and the other had a single nonmalignant cholangioma (Harshbarger 1997). None of these fish could be included in the biomarker assays because insufficient amounts of tissue were retained. External tumors, melanomas, and papillomas were only slightly more prevalent at BR (12 of 24 fish) than at OWC (10 of 30 fish). The general conclusion of the histopathology report on these fish (Harshbarger 1997) was that neither of the two groups exhibited significantly different neoplastic, parasitic, or subtle changes.

Sediment grab samples taken at the time of fish collection were extracted and analyzed for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) equivalents (TEQ) using the H4IIE cell-based assay (Sanderson et al. 1996). The crude extracts (i.e., not cleaned on sulfuric acid/silica gel) of BR sediment had $2,187 \pm 608$ pg/g TEQ whereas the OWC sediments had only 69 ± 20 pg/g TEQ. The cleaned extracts from either site had no more than background TEQs. The implication is that the BR sediment sample retained substantial PAH contamination.

A common response to xenobiotic stress is an increase in the production of detoxifying enzymes such as those associated with cytochrome P450 or antioxidant enzymes and protective substrates such as TGSH/GSSG. Table 1 shows the analysis of covariance for the antioxidant parameters of this study. Analysis of covariance provides a test of the difference between location means after adjusting for the effect of the covariate. Because RLW differed significantly with location and sex, it was included as the covariate for all the liver parameters. Catalase activity and TGSH were significantly greater in livers of OWC fish as compared to livers of BR fish. Mean SOD activity was significantly greater in the BR fish than in the OWC fish.

The enzyme and TGSH results compare with the results of Otto and Moon (1996) who found significantly higher SOD activity and significantly lower TGSH and CAT activity in brown bullhead livers from the contaminated St. Lawrence River than in livers of bullheads from the relatively uncontaminated Lac La Pêche. The trends in TGSH, SOD, and CAT appear to show that BR is relatively more contaminated than is OWC. The differences in adjusted means of the other antioxidant enzymes (GPX, GST) are not significantly different in livers from OWC and BR, but trend in the same direction as was the case in the Lac La Pêche/St. Lawrence River study, also indicating higher contamination of the Black River fish.

Three of the non-oxidative stress biomarkers measured in this study provide conflicting results: DNA strand breakage, EROD, and RLW. The DNA single strand break assay (ssDNA) detects damage caused by bulky DNA adducts; the more damage to the DNA, the more rapidly the DNA

Table 1
Analysis of Covariance Results for Biomarker Assays of Brown Bullhead Livers from Old Woman Creek (OWC) and the Black River, Ohio (BR)[1]

Analyte	Data Trans-formation	Location		Sex		RLW		Adjusted Mean[2]	
		F	P	F	P	F	P	F	P
CAT	None	10.01	**0.0069**[3]	0.24	0.6284	2.24	0.1565	**12.4**	5.81
CPX/CP	None	0.43	0.5221	2.77	0.1180	0.40	0.5389	261	299
CPX/HP	None	0.13	0.7257	2.74	0.1201	0.14	0.7139	293	314
GR	Rankit	0.67	0.4265	3.64	0.0772	0.00	0.9961	0.754	0.511
GST	Rankit	0.01	0.9137	12.36	**0.0034**	4.06	0.0634	10.7	11.1
Protein, ♀	None	6.36	0.0531	NA[4]		7.70	**0.0392**	17.8	21.8
Protein, ♂	None	2.97	0.1286	NA[4]		3.61	0.0991	21.5	17.9
SOD	None	8.21	**0.0125**	6.83	**0.0204**	2.29	0.1521	0.054	**0.077**
GSSG	Rankit	4.17	0.0606	0.25	0.6246	0.54	0.4745	0.008	0.005
TGSH	None	5.08	**0.0407**	7.11	**0.0184**	6.20	**0.0260**	**1.043**	0.801

1 Percent relative liver weight (RLW) used as the covariant, location as the primary factor, sex as the secondary factor of interest.
2 Units: Catalase (CAT) is reported as ln(A1/A2)/(t2-t1)*mg protein, where A1 and A2 are absorbance at 550 nm at times t1 and t2; Selenium-independent glutathione peroxidase (GPX/CP), selenium-dependent glutathione peroxidase (GPX/(HP), and glutathione reductase (GR) are reported as nmol NADPH consumed/min*mg protein; glutathione S-transferase (GST) is reported as µmol CDNB conjugated/min*µg protein. Protein is reported as mg/g tissue (wet wt); superoxide dismutase (SOD) is reported as mg for 50 percent inhibition of cytochrome c oxidation in the presence of xanthine oxidase; total glutathione (TGSH) and oxidized glutathione (GSSG) are reported as µmol/g tissue (wet wt).
3 Bold indicates significant, $P < 0.05$.
4 Not applicable. Anova showed significant location-by-sex interaction. Therefore each sex was analyzed separately.

will unwind under alkaline conditions, and the lower the resulting F-value. Results obtained in OWC and BR bullheads are shown in Table 2. The significantly lower F-value in OWC fish as compared to that of BR fish indicates more DNA damage at the OWC site. Significantly greater DNA damage would ordinarily be expected to result in significantly increased tumorigenesis, other factors being constant between the two populations. In fact, the number of fish with liver neoplasms was the same at the two sites and amounted to 6.7 percent of those sampled at OWC and 8.3 percent at BR. If the BR site is actually the more degraded, as appears likely based on its past history and most of the findings of this study, it may be that previous high exposure of the BR brown bullhead population to PAHs caused selection for fish with enhanced ability to repair DNA damage. An additional apparent anomaly that could be explained by constitutive differences in the metabolic capabilities of the two bullhead populations is the greater EROD activity in the OWC livers (Table 2). One would ordinarily expect to find EROD induction associated with PAH and/or PCB/PCDD/PCDF contamination. For example, bullhead livers had nearly threefold greater EROD activity in the St. Lawrence River than at Lac La Pêche (Otto and Moon 1996).

Table 2
Comparisons of Ethoxyresorufin-*O*-deethylase (EROD) Activity and DNA Single Strand Breakage (ssDNA) in Livers of Brown Bullhead at Old Woman Creek and Black River, 1996

Parameter	Old Woman Creek	Black River
ssDNA[1]	0.403 ± 0.0463 (6)[3]	0.673 ± 0.0665 (6)
EROD[2]	3.80, 2.74 to 4.91 (5)[4]	1.33, 0.945 to 1.41 (5)

[1] Mean ± SE (n). Calculated as F-value in the alkaline unwinding assay using Hoechst dye.
[2] Median, IQR (n). Units are ng resorufin/min*mg cytosolic protein.
[3] Significantly lower than Black River, $P = 0.008$ (t-test).
[4] Significantly higher than Black River, $P = 0.016$ (Mann-Whitney Rank Sum Test).

The marked differences in RLW (Table 3) also support the possibility that the Black River cohort have adopted a different mode for dealing with xenobiotics in their environment. There are consistent differences between the sites and between the sexes, with males and females at the BR site tending to have RLWs 36-38 percent greater than fish of the same sex at OWC, indicating enlarged livers at the BR location. This trend is consistent with the observations of Fabacher and Baumann (1985), who also described enlarged livers in BR fish relative to areas unimpacted by pollution. While still significantly enlarged, the BR RLWs measured in 1996 (this study) were only about 50-60 percent as large as reported in 1985. Liver enlargement indicates a greater than normal detoxifying capability due to the resulting higher absolute levels of protective enzymes and appears to be an adaptation to chronic conditions. The fact that this condition persists in the fish at BR after PAH levels in the sediments have subsided (Baumann and Harshbarger 1995) suggests that either the fish are still being stressed, although less than previously, or the adaptation has become a permanent characteristic of the population.

Increased RLWs in fish have been associated with contaminant stress in other studies and it has been suggested that RLW should be used as one of the factors in a battery of biological indicators of chemical contamination of water and contaminant stress in fish (Fabacher and Baumann 1985).

Table 3 Liver Weight as a Percentage of Body Weight (Relative Body Weight, RLW) in Brown Bullheads Collected at Old Woman Creek and in the Black River, Ohio, 1996		
	RLW	
Location	Males	Females
Old Woman Creek	1.696 ± 0.0349 (22)[1]	2.571 ± 0.0954 (8)
Black River	2.343 ± 0.139 (12)[2]	3.502 ± 0.249 (10)[3]
[1]Values are mean ± SE (n). [2]Significantly different than Old Woman Creek, $P < 0.001$ (t-test). [3]Significantly different than Old Woman Creek, $P < 0.006$ (t-test).		

CONCLUSIONS: Of the oxidative stress biomarkers included in this investigation, increased SOD and decreased CAT and TGSH appear to be most sensitive in brown bullhead. Responses of GR, GST, GSX, and GSSG have shown differences between fish from contaminated and uncontaminated sites in other studies, but no differences were seen in the present study. Results with ssDNA and EROD were the reverse of what has been reported in most other studies. There were no gross differences in pathologies detectable in fish from either site. The major difference found in bullheads at the two sites was in the size of their livers.

The fish at the Black River site appear to have developed responses to xenobiotics in their environment that depend on alterations in their absolute amounts of protective enzymes and substrates. This is evidenced by their enlarged livers, and is the type of adaptation that could have occurred through chronic exposures over generations. By contrast, fish from Old Woman Creek appear to retain the ability to respond to xenobiotics by inducible mechanisms, as is typical of organisms that do not live in a chronically and severely degraded environment. The ability of biochemical indicators such as these to serve as indicators of the health of recovering ecosystems appears to be limited by adaptations that may have occurred in populations previously exposed to high levels of contamination.

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